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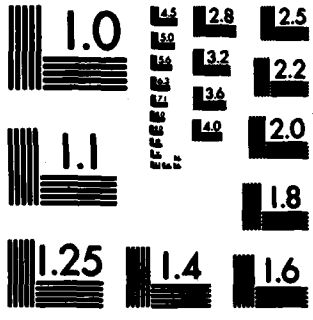

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Pseudomonas Flagella Vaccine in Burns

ANNUAL SUMMARY REPORT

Thomas C. Montie and Janice S. Allison

September 1, 1983

Supported by

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University of Tennessee
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Knoxville, Tennessee 37996

Foreword

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals", prepared by the Committee on Care and USP of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 78-23, Revised 1978).

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ABSTRACT

The focus of this research has been to investigate a Pseudomonas flagellar antigen (FAg) as a possible vaccine for prevention of Pseudomonas infections of wounds, burns and trauma associated infections. A second aim has been directed toward understanding the interaction of various Pseudomonads with the host with respect to the role of motility in colonization, invasion, and protection by a FAg induced immune system. Purification of the FAg has centered on reducing the level of LPS (the major contaminant) in the final material. Some progress has been made using a column-detergent method in conjunction with large fermentation facilities developed in collaboration with Immuno Co. We continue to explore and improve techniques of culturing the organism to reduce LPS and contaminating protein in the initial medium. Alternative approaches are described to find more efficient purification procedures. Our knowledge of the Pseudomonas aeruginosa a and b FAg types has been expanded significantly by creating a system of classification based on flagellin molecular weights which remains consistent with immunologic determinations and in preliminary experiments by protective capacity. An extension of this classification has been the finding that a taxonomic correlation exists between flagellin molecular weights of various Pseudomonas spp. and their antigenic relationships. In P. cepacia strains, two flagellins types (Types I and II) have been designated in a classification analogous to the P. aeruginosa a and b types. Protection studies in a burned mouse model (BMM) with partially purified FAg show that protection is obtained at challenge doses of 4-6 log CFUs over the LD_{50} of non-immunized controls. Results of studies with heated and polymyxin B treated FAg indicate that protection is caused by a specific FAg component. This conclusion is consistent with data showing that cross protection relates to the FAg rather than the O antigen component. Using the BMM we have compared the invasiveness of isogenic P. aeruginosa strains deficient in flagella to further

elucidate the role of motility in virulence. Lack of invasiveness by other Pseudomonas spp. was studied with emphasis on P. cepacia. Colonization of the burned site occured for three weeks without invasion. Since this organism is highly motile and chemotactic, the lack of invasiveness was attributed to the absence of protease and possibly Exotoxin A. This provides an interesting model to study chronic or persistent infections and to identify virulence factors. A single immunization (1 ug) of P. cepacia FAg did not block skin colonization to any significant degree although some protection was suggested. Discussions of the results and future directions are incorporated in the following text.

ANNUAL REPORT

A. Pseudomonas aeruginosa Flagellar Vaccine Studies

1. Relationship of molecular weights and flagella antigenic types

Extensive repetition of SDS-PAGE comparisons of molecular weights support the concept that flagellar antigenic (FAg) type can be classified by molecular weights of the flagellins (Table 1). These results have been submitted for publication to the J. of Clin. Microbiol. We have refined our gel techniques to obtain more accurate weight estimates by adding the same amount of FAg (one ug) to the well when making comparisons. Additional information has been obtained by co-electrophoresis of two antigens with very similar molecular weights. Thus we know that "a" type antigens are more electrophoretically mobile than "b" type. The "b" type antigens are a homogenous group of 53,000 mol. wt. while the "a" type are heterogenous with molecular weights from 45,000 to 52,000 (Table 1, Fig. 1). Of interest for possible vaccine application is that a significant proportion (close to 50% or more) of clinical isolates have "b" type flagella (Lanyi (1) & Ansorg (2)). We have observed a similar distribution of types among our laboratory strains and clinical isolates. Of the unidentified "a" types, we have demonstrated that strain 1210 and 19660 (Berk's eye strain) both virulent isolates, are identical to 5933 (A_{012}) (Table 1 and Fig. 1). The 1210 identity has been further identified by cross-agglutination reactions both in our laboratory (Table 2) and by Immuno Co. of Austria. On the other hand WR-5 (avirulent) and GNB-1 (virulent) remain to be antigenically classified. Although they exhibit similar molecular weights (WR-5 = 48,000 and GNB-1 = 49,000) to 5940 (A_{02}) (47,000) they can be separated from 5940 by co-electrophoresis (Fig. 2).

Two discrepancies between our work and that of Ansorg (2) have been noted. A novel finding is that FAg of Ansorg's type "a_0" strains exhibit the same

molecular weight as the "b" strains (Fig. 3). This may be an error in his original typing experiments (Ansorg, personal communication) or it may mean that a$_0$ = b and therefore that common antigens are present in all a types and b types. This is an important question to be resolved by further biochemical comparisons. Secondly, Ansorg assigned 7191 a b type, however we have found a molecular weight of approximately 51,000, putting it in the a classification similar to 5933 (Fig. 3).

2) Approaches to purification of flagella antigen

a) Shearing methods. Attempts were made to obtain more pure FAg preparations free of outer membrane components by reducing the length of time needed to initially remove flagellar filaments. Our published method (3) based on several literature references for other gram negatives was to shear for 3 min. in a blender. However, small but consistent amounts of LPS (5 to 15%) remained associated with the FAg preparations. Strain M-2 was used as the standard culture and suspensions were sheared at various times to determine the effect of shearing on protein yields and LPS content. Initial experiments indicated that 1 min. gave the same results as 3 min. Bacteria were then sheared for 15, 30 and 60s. In all cases however, we were not able to exclude LPS to any significant degree. The protein content ranged from 30 to 50% of dry weight based on an albumin standard. By a globulin standard in the BIORAD assay this would be closer to 60 to 100%. Further experimentation has shown that LPS is consistently present in the medium during growth of P. aeruginosa and that it probably binds to the flagellar filaments before they are sheared. Constant sloughing of LPS during rapid growth has been reported very recently by Cadieux et al. (4). Since the LPS is not removed during differential centrifugation it must be separated by some detergent process from the flagellar protein (T. C.

Montie et al., unpublished data). Attempts to wash the bacterial cells before blending using buffer, water or EDTA does not reduce the LPS content to any extent.

b) Removal of LPS

As discussed above, removal of LPS has more recently become of prime concern to us for both obtaining a product that could be injected into humans with no side reactions and for eliminating unknown biologic effects in animal tests. One preliminary approach has been to use the method of Parenchych (5) who employed octyl-glycopyranoside (OGP) to dissociate Pseudomonas pili. Flagellin were dissociated in 0.1% OGP followed by dialysis to remove impurities. Electrophoretic assay indicated that the flagellin remained in a native configuration but a non-distinct broad band, possibly dissociated LPS, appears. We plan further experimentation to utilize ultracentrifugation in OGP in buffer or in density gradients to separate the dissociated flagellin from LPS. Micro-molecular sieving column methods will be employed concurrently to test the degree of flagella dissociation relative to the LPS size.

As mentioned above a detergent molecular sieving column method is being worked out in collaboration with Immuno Co. This method holds potential for separation of FAg from LPS effectively and on a relatively large scale. On a smaller scale we have continued to increase yields of flagellin eluted from gels after resolution by SDS-PAGE. By this approach we should simultaneously remove both carbohydrate and protein impurities.

c) Removal of protein impurities

Another method used for separation of flagella from pili of P. aeruginosa (6) was employed to isolate flagella from supernatants of sheared P. aeruginosa cells (200 g wet wgt. cells). Polyethylene glycol 6000 (PEG-6000) 0.1% was added sequentially 3x to the supernatants to obtain a protein precipitate.

Protein profiles of the washed precipitate indicated that a number of small molecular weight contaminating proteins had been removed, but not LPS. We plan to use a combination of this method with a detergent to concomitantly remove both LPS and protein impurities.

Although protein impurities appear as <5% of the total protein, we observed the presence of two to three small molecular weight proteins (15,000 to 20,000) appearing as prominent bands in gel profiles of FAg's of a_0 a_1 a_2 type such as 5933. This protein appears to be dissociated flagella (possibly protease nicked flagella) and/or pili material (17,000 mol wgt (7)). With 5933 FAg we have noted increased amounts of some of these bands and decreased amounts of native flagellin correlated with length of time in storage. Reduction in the apparent degradation has been obviated by shifting from storage after lyophilization in weak phosphate buffer to storage in a Tris-Cl buffer (pH 7.5, 0.01 M) (see Fig. 1).

An interesting result along this same line is that growth of P. aeruginosa in well aerated cultures yields FAg preparations exhibiting very low amounts of a 19,500 mol. wt. band, but no. 17,500 band (pili-like band) (Fig. 4). In contrast growing cells in reduced O_2 atmosphere gives a relatively larger amount of both of the small molecular weight bands and a reduced yield of flagellar material (Fig. 4). We plan to explore this approach to maximize protein purity in the FAg a_0 a_1 a_2 type.

3) Protection with Flagellar Antigen

a) Experiments with heated FAg

We have extended our protection studies in burned mice using additional mouse strains such as C3H/HEJ and HA/ICR. Protection was obtained, using 1 ug M-2 FAg injected two weeks before challenge, at a level up to 15,000

LD_{50}'s (LD_{50} = >> 2.3 x 10^5 CFU). Heated FAg (90°C 1 h) lost nearly all of its protective capacity (LD_{50} = <2.3 x 10^2 CFU, Table 3 Appendix). This result underscores that the immunogenic specificity resides in the protein FAg present rather than in contaminating material such as lipopolysaccharide (LPS). It should be noted that results obtained at Immuno Co. indicate that more highly purified FAg may protect at a level of at least 6 logs CFU challenge above the LD_{50}.

We have carried out preliminary experiments comparing FAg extracts from our M-2 Fla$^-$ strain after boiling versus non-boiled. Although we expected no differences in protection in this case since the majority of material in the FAg (Fla$^-$) pellet is LPS some loss of protection was indicated (approximately 1 log LD_{50} of CFU) (Table 3). Subsequent careful SDS-PAGE analysis of the Fla$^-$ FAg preparations indicated that a small amount of protein banded as M-2 flagellin at 53,000 daltons. It appears that in the M-2 Fla$^-$ bacteria some flagellin monomer is made and accumulated perhaps in the membrane (8), but an intact flagellum cannot be polymerized. We plan repetition of this work using other Fla$^-$ extracts of other strains (see discussion below).

b) FAg Immunization in C3H/HEJ; mice non-responsive to E. coli LPS

Experiments were carried out using M-2 FAG to immunize C3H/HEJ mice. These mice are refractory to E. coli or salmonella LPS, (9), but are partially stimulated in their immunologic response by P. aeruginosa LPS (Pier (9)). Immunization of these mice with 1 ug M-2 FAg provided scattered protection from 10^2 to 10^5 CFU (Table 4). However, heating the antigen for 1 h at 100° completely destroyed the immunogenicity (LD_{50} = < 10^2). We believe that the scattered protection achieved with non-heated FAg is indicative of the decreased protection expected in C3H/HEJ mice with LPS (and perhaps FAg) (Pier (9)). The decreased protection with heated FAg again suggests that the

flagellar protein is responsible for protection. These experiments will be repeated using C3H/HEJ mice immunized with both pure LPS and FAg obtained from the same P. aeruginosa strain (ATCC 27312). Challenge will be after five days immunization (Pier experiments) or two weeks (our experiments). Again heated and non-heated FAg preparations will be compared.

An experiment related to those discussed above will involve testing the effect of polymyxin B on the FAg and isolated LPS in C3H/HEJ since Pier et al. (9) have reported that the peculiar lipid A portion of P. aeruginosa LPS is primarily responsible for the immune stimulation in these mice. It is well known that polymyxin B specifically inactivates the lipid A portion of LPS and this has been recently demonstrated for Pseudomonas LPS. In our hands using HA/ICR LPS responsive mice, 30 minute treatment of M-2 FAg with polymyxin B had no apparent effect on the immunoprotection of this preparation (Table 5). Although this result suggests that the lipid A portion of LPS is not contributing to protection of the FAg preparation, this experiment needs to be repeated with additional controls (for example, P. aeruginosa LPS alone, polymyxin B treated and untreated). In selected experiments we also plan to monitor the immunogenic response in mouse strains during the five day and two week periods.

B. Pathogen-Host Interactions with Regard to Motility and Chemotaxis and Flagellar antigen

1. The role of flagella; motility and chemotaxis in virulence.

To understand the interplay between P. aeruginosa and the host with respect to flagellar vaccine protection we have continued to pursue studies on bacterial cell movement in the host. Evidence to date from studies examining the role of flagella and motility as virulence factors suggest that an association of these bacterial properties with virulence does exist (McManus (10), Montie et al. (11)). Thus we have begun using isogenic strains of virulent P. aeruginosa such as M-2 to understand the mechanism of invasion in the burned mouse model (BMM). A non-motile mutant M-2 Fla$^-$ was three to four logs less virulent than the parental strain, whereas a spontaneous motile revertant regained full virulence. We have recently repeated this study with similar results using two additional mouse strains.

The invasive character of P. aeruginosa is particularly important in burns and trauma infection where bacteremia leads to organ invasion and death. Thus the success of a flagellar vaccine may relate to the activity of antibody (opsonizing or otherwise) in effectively reaching the bacterial flagellum and severely immobilizing the bacterial cell.

Since we have only demonstrated the apparent Fla requirement for a single mutant we have extended these studies. In addition to the experiments with M-2 Fla$^-$ we have obtained mutants of PAO-1 from Hancock-Kropinski and are comparing them with respect to their isogenic character. Preliminary studies indicate that these strains are good candidates to further evaluate the role of flagella as a virulence factor. Strains PAO-1 WT, and three AK mutants (Fla$^-$) have been compared with regard to O serotype (all are serotype 5 with weak reactions to type 7 antisera), protease (all are highly proteolytic) and growth rates (similar growth curves).

Antibiogram profiles are being made. The AK mutants, obtained from a well characterized PAO-1, WT, (LD_{50} = $<10^2$ CFU), should provide evidence to further substantiate that flagella (motility) is important in the invasive role of P. aeruginosa. Initial comparisons of PAO-1 with the EMS derived AK mutants indicates that the AK mutants are greatly reduced in virulence with doses as high as 10^7 CFU required for 0-40% mortality in the BMM. These virulence tests are currently being repeated with larger numbers of mice.

The Tsuda-Iino strains (13) have been recently obtained and are being investigated. These are genetically constructed flagellar-minus (fla^-), motility-minus (mot^-) and chemotaxis-minus (che^-) strains. They are currently being characterized for their isogenicity and will be used in comparative virulence studies by skin surface inoculation as well as by inoculations from different sites eg. i.p. alone, and after immunization with FAg vaccine. This will allow us to extend our insight into the role of P. aeruginosa movement in pathogenesis. The parent WT 1200 is virulent in our model (LD_{50} = 10^2 CFU). Other sets of strains such as a Che^+ mutant of WT Che^- WR-5 (LD_{50} = 10^7 CFU) and a series of "rough" cystic fibrosis (CF) Pseudomonas aeruginosa isolates which are avirulent in the BMM (LD_{50}'s = $>10^4$ CFU) will provide valuable comparisons. The CF strains are being characterized morphologically and biochemically. A new finding is that 70% of these rough isolates from patients in poor clinical condition are Fla^- (M. A. Luzar & T.C. Montie, manuscript in preparation). The latter finding suggests that a Fla^- characteristic may contribute to "non-irvasiveness" or chronic infection.

Other approaches planned will include comparing the virulence of these mutants using burns of longer and shorter durations. This will include also comparisons in scalded mice (10). The object will be to assess movement through

various kinds of impaired tissue in compromised FAg immunized animals. Tissue colonization will be assayed by bacterial cell counts (12) and by sectioning and staining tissue to assess spreading and degree of penetration.

2) Host Response

We continue to collect antisera both from hyperimmunized mice and rabbits (12). This sera has been used for agglutination assays and for immunodiffusion assays. We have confirmed for the most part the relationship between flagellan antigen type (2) and molecular weight (see previous discussion). In the same way we have identified virulent clinical isolates corresponding to known antigen strains. When we have obtained monospecific serum using more highly purified FAg we will use this material to do comparative cross-protection tests with strains of homogenous and heterogenous antigenicity using actively and passively immunized mice.

Our studies examining the role of motility in infection will be interphased with studies focusing on inhibition of motility in vitro with specific anti-flagellar antiserum and phagocytosis studies. Preliminary experiments indicate that antisera raised in mice and rabbits (single and multiple antigen challenges) will inhibit motility as assayed by a filter disc-soft agar plate system. Paper discs saturated with 10-fold antisera dilutions significantly reduced spreading as compared to normal serum controls.

C. Studies with Nosocomial Pseudomonas spp.: Flagella Characterization and Protection and P. Cepacia Colonization.

We indicated in our first contract proposal our interest in studying the non-aeruginosa Pseudomonads and comparing their flagella antigens with P. aeruginosa. In fact a significant degree of effort has gone into this research area. It is apparent that the appearance of the Pseudomonas spp. as nosocomial agents is increasing (14). In many cases the source of the infections are contaminated hospital liquids or instruments which introduce the organism directly into the compromised patient, many times intravenously. Quite commonly these Pseudomonads are more resistant to antibiotics than P. aeruginosa and therefore they persist after chemotherapeutic treatments. An example of their opportunistic nature can be seen in the severe and fatal lung infection caused by P. cepacia in cystic fibrosis patients. In many cases the P. cepacia appearance follows a previous chronic P. aeruginosa infection, but the cepacia infection leads to a rapid decrease in the patients condition. In fact, P. cepacia is probably the biggest problem of the non-aeruginosa species, and we have therefore concentrated our approach on this organism.

1) Isolation and characterization of flagellar preparations from Pseudomonas species.

Some studies were directed toward isolating the FAg from both P. cepacia and other Pseudomonads to compare them with P. aeruginosa (14). An initial survey of FAg was made of P. cepacia, P. maltophila and P. stutzeri. P. stutzeri flagellin, mol. wt. of 55,000, was somewhat larger but close to the P. aeruginosa b type. P. maltophila flagellin was much smaller, 33,000, whereas P. cepacia SMH exhibited the lowest size, 31,000 (Fig. 5). The sample size of P. cepacia was increased because of its prominence as a nosocomial pathogen and

its wide distribution. Five additional clinical strains were examined. The distribution of molecular weights indicated a division into two groups. Flagellins with molecular weights of 31,000 were designated as Type I flagellin while the second group, designated as Type II flagellin, was in the 44,000 to 46,000 range. Further support for categorizing P. cepacia flagellin into either Type I or Type II was obtained by examination of four additional clinical isolates (Figure 6). These four could also be placed in either Type I or Type II based on their flagellin molecular weight, one was Type I while three were Type II. It was noted that all Type I flagellin samples exhibited a double-banded pattern on the gels (Figures 6, 7, and 8). This was observed consistently with all three Type I isolates identified (i.e., SMH, E7427 PR, F3761 PR).

Further evidence supporting segregation of P. cepacia flagellins into two types was obtained by determining their serological relationships. This approach has been used succesfully in categorizing the flagellins of P. aeruginosa (1, 2, 15, 16). Hyperimmune antisera prepared against b, a_{012}, and a_{034} FAg of P. aeruginosa would not react with any of the non-aeruginosa pseudomonads (Table 6). Antisera prepared against P. cepacia SMH (Type I FAg) would agglutinate only P. cepacia strains with Type I FAg (Table 7). Antisera prepared against P. cepacia E8980(1) Col (Type II FAg) would agglutinate only P. cepacia strains E8980(1) Col and 3765 Pa (Type II FAg); the other five P. cepacia strains with type II FAg were not agglutinated (Table 7). A similar situation is seen with antisera prepared against P. cepacia E4119 Ca (Type II FAg) and D7072 Ind (Type II FAg). These antisera would agglutinate only P. cepacia strains E4119 Ca and D7072 Ind; the remaining strains with Type II FAg were not agglutinated (Table 7).

The serological evidence is consistent with the molecular weight results and the conclusions that a common antigenic relationship exists among Type II flagellins but not between Type I and Type II flagellins. Some homology is shown among Type II flagellins.

As pointed out earlier, flagellin isolated from different strains of Pseudomonas aeruginosa differ antigenically as detected by cross agglutination (1) and indirect fluorescent antibody technique (15). Lanyi (1) and Ansorg (2) have reported that there are two major types of flagellin among strains of P. aeruginosa: type "a" shows a complex antigenic structure with several combinations of subtypes a_0, a_1, a_2, a_3, and a_4; type "b" shows a uniform antigenic structure (2). These differences in P. aeruginosa flagellin types have also been demonstrated electrophoretically. Type "b" flagellins have a molecular weight of 53,000 while the "a" type flagellins range from 43,000 to 52,000 (3, 17).

Results obtained in this investigation demonstrate that there are also major differences between the flagellin molecular weights of various Pseudomonas species. The large variation in molecular weights between Pseudomonas species is not surprising since the species studied are taxonomically distant (18, 19, 20). This taxonomic relationship is supported by the fact that hyperimmune antisera raised against P. aeruginosa flagellin types (b, a_{012}, a_{034}) would not agglutinate any other Pseudomonas species. By various criteria, P. stutzeri is taxonimically close to P. aerugionsa (18). Of the five Pseudomonas RNA homology groups, both P. stutzeri and P. aeruginosa are in RNA homology group I (19, 20). This taxonomic relationship may be further reflected by P. stutzeri flagellin having a molecular weight similar to the b flagellins of P. aeruginosa. Pseudomonas maltophilia and P. cepacia are taxonomically distant

from both P. aeruginosa and P. stutzeri. Pseudomonas cepacia is in RNA homology group II while P. maltophilia is in group V (18, 19, 20). This may also be reflected by the dissimilarity of the molecular weights of their flagellins in comparison to the flagellin of P. stutzeri and P. aeruginosa. It is interesting to note that the complex flagella of P. rhodos (a nomenspecies of Pseudomonas that has been incompletely described) are composed of flagellin with a molecular weight of 55,000, identical to that of P. stutzeri (18, 21). This raises the possibility that P. rhodos might one day be placed in RNA homology group I along with P. stutzeri if flagellin molecular weights correlate with RNA homology.

A more detailed examination of P. cepacia isolates revealed that flagellin from P. cepacia strains could be separated into two distinct groups. Type I exhibited a molecular weight of 31,000; Type II had molecular weights of 44,000 to 46,000. The molecular weight relationships apparently are reflections of antigenic relationships since hyperimmune antisera raised against P. cepacia Type I flagellin will only agglutinate Type I cells; no cross-reactivity with Type II strains was observed. However, hyperimmune antisera raised against P. cepacia strain E8980(1) Col (Type II FAg) would agglutinate two of the seven type II strains, E8980(1) Col and 3765 Pa. Hyperimmune antisera prepared against P. cepacia E4119 Ca (Type II FAg) and P. cepacia D7072 Ind (Type II FAg) would also agglutinate either E4119 Ca or D7072 only; however, neither would agglutinate any of the remaining five Type II strains. The lack of cross-agglutination may suggest that there are small differences in Type II flagellins analogous to that observed with the various P. aeruginosa "a" flagella antigen subtypes (2). These Type II molecular weight and serological differences may be due to slight differences in amino acid sequence and/or arrangement. The uniform "b" type of P. aeruginosa may be an analogous

situation to Type I of P. cepacia since both groups clearly contain, within their own group, flagellins of homologous molecular weight and antigenicity.

Although Type I flagellins of P. cepacia do show homology, there is a double banded pattern in these flagellins. This observation suggests that either two monomeric flagellins of nearly identical molecular weight exist in each flagellum or that two closely related proteins are associated with at least two different flagella present on P. cepacia Type I cells. A two component flagellin system is common among the uniflagellated Caulobacters. Seven strains have been found to have two flagellins that differed by only 1,000 MW (22). The two flagellins of Caulobacter crescentus CB13B1a (differing by only 3,000 MW) are immunologically identical (23). Pseudomonas rhodos produces two types of flagella: complex flagella have 55,000 MW flagellins, while the plain flagella have 37,000 MW flagellins (21). The latter type of flagella are separable into two slightly different monomers (21). Pseudomonas cepacia has been shown to possess more than one flagellum (18, 24). These can be in a polar multitrichous or lateral arrangement (25).

In a study by Johnson (26), 74 isolates of P. cepacia had 1 to 4 polar flagella and 30 isolates had 5 to 8 polar flagella. Thirteen isolates exhibited two types of flagellation, the normal wavy polar flagella and tight curly lateral flagella. A plausible explanation of the double banded pattern is the possibility that flagellin dimers were disassociated due to the electrophoretic procedure. This explanation is unlikely, however, since no "native" dimeric form (of approximately 60,000 MW) is observed on the gels. More data are needed to determine the exact flagella composition of Type I strains.

Characterization of P. cepacia strains into two flagella antigen molecular weight types may have merit as a possible epidemiological tool. The

categorization may serve a role similar to that proposed for other bacteria (e.g., outer membrane proteins of Neisseria meningitidis, flagella of Serratia mercescens and Pseudomonas aeruginosa (17, 27, 28).

2) Virulence of Pseudomonas spp. in a burn mouse model: tissue colonization by Pseudomonas cepacia.

Although P. aeruginosa is not very virulent in normal animals, it becomes extremely virulent in compromised animals such as burned mice (29). Comparison of other pseudomonads showed in contrast little lethality until $\geq 10^7$ CFU were injected. Topical application produced no deaths at 5×10^7 CFU. In the same animal model P. aeruginosa M-2 was 100% lethal at 10^2 CFU. Similar to the non-aeruginosa pseudomonads E. coli injection resulted in only 10% lethality as has been previously reported (30).

Pseudomonas aeruginosa is a highly invasive organism in compromised hosts. Within 28 h. of subcutaneous injection of 10^2 CFU of P. aeruginosa strain M-2 into the burn site, the bacteria rapidly multiplied with 10^8 CFU/g detectable in the burned skin (Fig. 9), 10^5 CFU/g liver and 10^3 CFU/ml blood. This data confirms previous studies. Although less virulent, P. cepacia SMH colonized the burned skin of thermally injured mice. When 10^5 CFU of P. cepacia SMH was injected subcutaneously into the burn site, the levels of P. cepacia increased within 24 h to 10^7 to 10^8 CFU/g burned skin and persisted at this level for at least 17 days (Fig. 10). When a lower level (10^2 CFU) of P. cepacia was injected a similar pattern was observed except that the highest level obtained was 10^4 CFU/g burned skin (Fig. 11). Similar colonization patterns were observed with another cepacia isolate strain E7427 PR. With strain SMH burned skin was colonized for three weeks before declining by four weeks post injection (Fig. 12).

Although colonization was prevalent no evidence was found for invasion by P. cepacia into the blood, liver, or unburned skin. P. cepacia did not colonize unburned skin when injected s.c. in burned skin of colonized mice. Swabs of both burned and unburned skin revealed no P. cepacia present on the skin surface of the majority of mice, however, a few mice had $\leq$ 80 CFU on the skin surface.

Mice immunized with P. cepacia SMH FAg (Type 1) did not give significant reductions in the number of homologous P. cepacia SMH colonizing the burned skin upon challenge (Fig. 13). Likewise, there was no difference in colonization by P. cepacia SMH in burned skin of mice that had been immunized previously with FAg of a heterologous P. cepacia strain E8980(1) Col (Type II), P. aeruginosa M-2 (Type b), or saline control (Fig. 13).

To explain differences in invasiveness between P. aeruginosa M-2 and the various Pseudomonas spp., protease assays were carried out (Table 8). All the non-aeruginosa species gave a negative test for protease compared to P. aeruginosa M-2, the virulent standard strain. It has already been reported that Pseudomonas spp. lack exotoxin A (31, 32). Since in particular proteases have been implicated as virulence factors (33, 34) we suggest that the absence of proteases may account for the lack of invasion of tissues and subsequent low mortality (12). Thus the rapid growth and spreading of motile organisms would depend on the ability to obtain nutrition from the burned wound which would be enhanced by extracellular enzymes. High inoculum numbers and/or rapid growth would increase chances of overcoming host defences at the burned site. Experiments are currently planned to examine the role of proteases in P. cepacia invasion by injecting protease with P. cepacia or by co-injecting P. cepacia and protease producing, but avirulent, P. aeruginosa such as strain WR-5.

This ability of Pseudomonas spp. to colonize and persist in a trauma wound may be significant. It is known that pseudomonads and numerous other Gram-negative bacilli are very opportunistic in their ability to infect compromised hosts (35, 36, 37). In a hospital environment, the extreme antibiotic resistance of some Pseudomonas spp. invariably results in selective colonization by these organisms. Opportunistic infections caused by non-aeruginosa pseudomonads are difficult to manage and may require frequent and sometimes toxic antibiotic treatment. Thus, the ability of these organisms to tenaciously persist in burn wounds may provide a model reflecting on the persistence of nuisance infections in compromised patients.

Holder and Montie recently demonstrated a significant level of protection against normally lethal P. aeruginosa burn infection by immunizing mice with a flagellar vaccine (33). Mice so protected had approximately 50% reduction in the number of bacteria in the liver; however, bacterial counts at the burned skin were unchanged (33). In these studies mice immunized and challenged with the homologous P. cepacia strain SMH (Type I FAg) also did not have a significant decrease in numbers of bacteria persisting in the burn wound. No differences were seen with other immunogens and heterologous challenge. Thus, although immunization with flagella significantly protects against lethal infection by blocking the invasive capacity of P. aeruginosa, such treatment has no effect on the initial proliferation and persistence at the burn wound by either P. aeruginosa or P. cepacia. Burn wound sepsis consists of two major phases: proliferation and invasion (38, 39, 40). Studies examining the role of motility of Pseudomonas spp. in the transition between these two phases are currently in progress, along with competition experiments involving P. aeruginosa and other Pseudomonas spp. in the burned mouse model.

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APPENDIX

List of Tables

Table 1. Comparisons of known H antigens of P. aeruginosa with H antigens of various unknown strains

Strain[a]	O-Antigen[a]	Antigenic component(s)[b]	Flagellin relative mol. wt.[c]	Unidentified Strains[c]
170001	3	b	53,000	M2,1244, PAO (PJ108 M446, etc.) SBI-I
15084	10	b	"	SBI-B, SBI-G, (O serotypes[d] B,D,G)
5142	16	a_0	53,000	-
13030	5	a_0	"	-
5939	1	$a_0.a_3$	52,000	-
5933	8	$a_0.a_1.a_2$	51,000	1210, 19660, 7191?
-	-	-	-	WR5(48,000),GNB-1 (49,000)
5940	6	$a_0.a_2$	47,000	
170018	-	$a_0.a_3.a_4$	45,000	-

a. Obtained from R. Ansorg, based on IATS standard typing.

b. These antigenic types were differentiated using the indirect fluorescent antibody technique.
Antigen a is characterized by the factor a_0 which is common to all "a" flagella, and the independent partial factors, a_1, a_2, a_3, a_4. The serotype of flagella bearing the complex antigen a are determined, therefore, by the common factor a and the partial factors a_1-a_4 in various combinations.

c. These strains are classified according to a similar or the same mobilities on SDS-PAGE. A question mark indicates we have not matched the unknown with a known antigenic strain.

d. O serotyping method from Homma et al., Japanese Journal of Experimental Medicine Vol. 47 No.3 p. 195-201, 1977.

Table 2. Cross-agglutination reactions using FAg antisera[a]

Antisera[b]	P. aeruginosa strains									
	M-2 (b)	SBI-B (b)	SBI-I (b)	SBI-G (b)	170001 (b)	13030 (20)	GNB-1 (a?)	WR5 (a?)	5933 ($a_0a_1a_2$)	170018 ($a_0a_3a_4$)
M-2 (b)	++	++	-	+	-	++	-	-	-	-
170001 (b)	++	++	++	++	++	-	-	-	-	-
5933 ($a_0a_1a_2$)	-	-	-	-	-	-	-	-	++	++
170018 ($a_0a_3a_4$)	-	+	-	+	-	-	-	-	+	++

a. Agglutination assays were performed as follows: turbid suspensions of bacteria from TSA plates were made in 0.85% saline. A 45 µl drop of 10x-diluted antisera was added. The slides were then rocked gently and continuously monitored for agglutination for 2 minutes and graded as follows:

++: strong agglutination
+: moderate-weak agglutination
-: no agglutination

Antigenic component(s) are in parentheses.

b. Rabbits were immunized with FAg according to a modification of the procedure of Lagenaur et al. (64). Six injections of FAg were given over a 29 day period followed by ear vein bleeding one week later.

Table 3. Comparison of protection in burned HA/ICR mice using native or denatured PAg

Immunogen[a]	M-2 Challenge[b] (CFU)	Mortality[c]
Boiled PAg	2.3×10^5 2.3×10^4 2.3×10^3	5/5 5/5 4/5
Non-boiled PAg	2.3×10^5 2.3×10^4 2.3×10^3	0/5 1/5 1/5
Control	2.3×10^5 2.3×10^4 2.3×10^3	5/5 5/5 5/5

a. Immunized I.M. with 1 ug M-2 PAg, boiled or non-boiled, or 0.1 ml 0.85% saline for control.

b. Strain P. aeruginosa M-2 challenge was 14 days following injection.

c. Cumulative mortality in a test group of five mice scored over a seven day period.

Table 4. Comparison of protection in burned C3H/HeJ mice using native or denatured PAg

Immunogen[b]	M-2 Challenge[c] (CFU)	Mortality[d]
Boiled PAg	2.6×10^5 2.6×10^4 2.6×10^3	5/5 5/5 5/5
Non-boiled PAg	2.6×10^5 2.6×10^4 2.6×10^3	2/5 1/5 2/5
Control	2.6×10^5 2.6×10^4 2.6×10^3	5/5 5/5 5/5

a. These mice are non-responsive to E. coli and Salmonella LPS.

b. Immunized I.M. with 1 ug M-2 PAg, boiled or non-boiled, or 0.1 ml 0.85% saline for control.

c. Strain P. aeruginosa M-2 challenge was 14 days following injection.

d. Cumulative mortality in a test group of five mice scored over a seven day period.

Table 5. Comparison of protection in burned HA/ICR mice using polymyxin B treated or untreated PAg[a]

Immunogen[b]	M-2 Challenge[c] (CFU)	Mortality[d]
M-2 PAg	7.7×10^4 7.7×10^3	1/5 0/5
M-2 PAg + Polymyxin B	7.7×10^4 7.7×10^3	1/5 2/5
M-2 Flg- PAg	7.7×10^4 7.7×10^3	3/5 2/5
M-2 Flg- PAg + polymyxin B	7.7×10^4 7.7×10^3	3/5 1/5
Polymyxin B	7.7×10^4 7.7×10^3	5/5 4/4
Non-immunized	7.7×10^4 7.7×10^3	5/5 4/5
Saline Control	Non-challenged	0/5

a. PAg treated by incubating with polymyxin B for 30 mins (RT) at a ratio of 1 ug PAg:10 ug polymyxin B.

b. Immunized I.M. with 1 ug M-2 or M-2 Flg- PAg (treated or untreated), 10 ug polymyxin B in 0.1 ml 0.85% saline, or 0.1 ml 0.85% saline as control to test lethality of burn itself.

c. Strain P. aeruginosa M-2 challenge was 14 days following injection.

d. Cumulative mortality in a test group of five mice scored over a seven day period.

Table 6. Slide Agglutination Assays of Pseudomonas Species with Antisera Against P. aeruginosa PAg.

Agglutination with viable cells	Antisera		
	P. aeruginosa 5933[a]	P. aeruginosa 170018[b]	P. aeruginosa 170001[c]
P. cepacia BNB	-	-	-
P. cepacia E4119 Ca	-	-	-
P. cepacia E8980(1) Col	-	-	-
P. cepacia E7893 Ill	-	-	-
P. cepacia E2973 Mo	-	-	-
P. cepacia E7427 PR	-	-	-
P. cepacia D7072 Ind	-	-	-
P. cepacia A1560 Fla	-	-	-
P. cepacia 3765 Pa	-	-	-
P. cepacia F3761 PR	-	-	-
P. maltophilia B69 Fla	-	-	-
P. stutzeri BNW	-	-	-

[a] P. aeruginosa PAg type 4912.

[b] P. aeruginosa PAg type 4934.

[c] P. aeruginosa PAg type b.

Table 7. Slide Agglutination Assays of Pseudomonas Species with Antisera Against P. Cepacia PAg.

Agglutination with viable cells	Antisera			
	SMH[a]	E8980(1) Col[b]	E4119 Ca[b]	D7072 Ind[b]
P. cepacia SMH[a]	+	-	-	-
P. cepacia E4119 Ca[b]	-	-	+	+
P. cepacia E8980(1) Col[b]	-	+	-	-
P. cepacia E7893 Ill[b]	-	-	-	-
P. cepacia E2973 Ma[b]	-	-	-	-
P. cepacia E7427 PR[a]	+	-	-	-
P. cepacia D7072 Ind[b]	-	-	+	+
P. cepacia A1560 Fla[b]	-	-	-	-
P. cepacia 3765 Pa[b]	-	+	-	-
P. cepacia P3761 PR[a]	+	-	-	-
P. maltophilia D69 Fla	-	-	-	-
P. stutzeri NEW	-	-	-	-

[a]P. cepacia PAg type I.

[b]P. cepacia PAg type II.

Table 8. Proteolytic Activity of Pseudomonas Species.

Organism	Proteolytic Activity[a]
P. aeruginosa M-2	+
P. cepacia SMH	-
P. cepacia E4119 Ca	-
P. cepacia E8980(1) Col	-
P. cepacia E7893 Ill	-
P. cepacia E2973 Ma	-
P. cepacia E7427 PR	-
P. maltophilia D69 Fla	-
P. stutzeri NEW	-

[a]Assayed on dialyzed brain heart infusion milk/agar plates.

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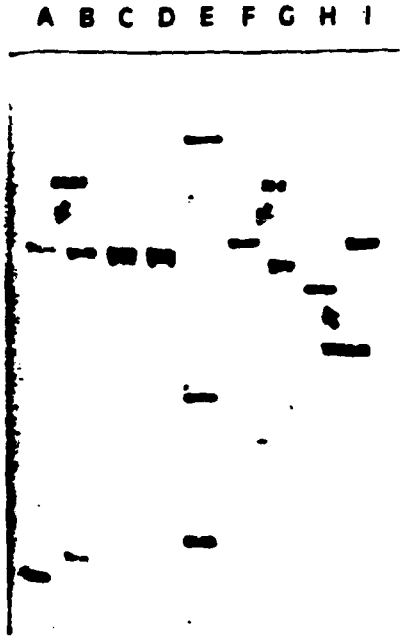


Figure 1. SDS-PAGE of P. aeruginosa FAg preparations. (Lane A) 5933 ($a_{0a_2}s_2$). (Lane B) 1210 ($a_{0a_1}s_2$). (Lane C) 1210 ($a_{0a_1}s_2$) Tris-Cl storage. (Lane D) 1210 ($a_{0a_1}s_2$) Tris-Cl lyophilized storage. (Lane E) Standard proteins and their molecular weights. From top, phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). (Lane F) M-2 (b). (Lane G) GMB-1 (a?). (Lane H) 170018 ($a_{0a_{24}}$). (Lane I) SB1-6 (b). 1 ug FAg loaded per well.

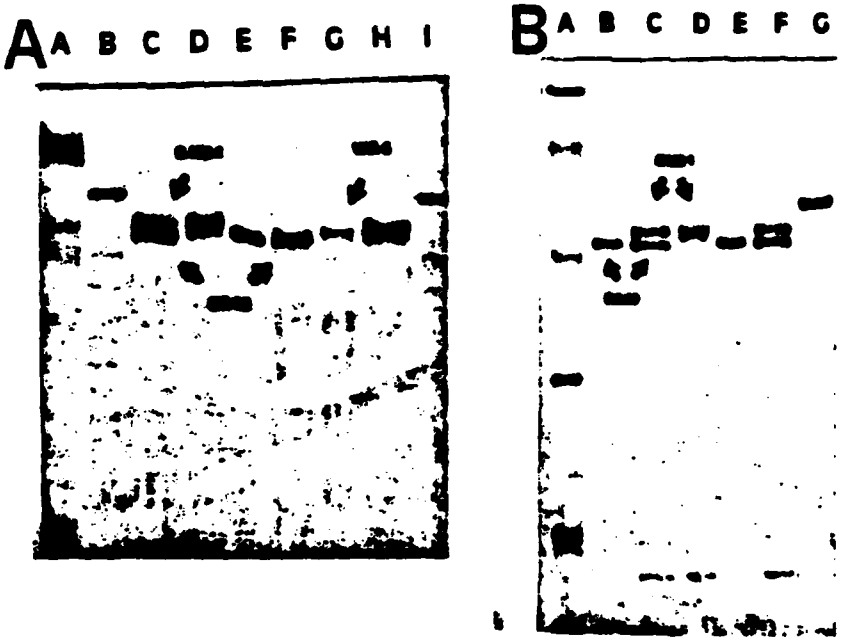


Figure 2. SDS-PAGE of P. aeruginosa FAg preparations. A (Lane A) Standard proteins and their molecular weights. From top, bovine serum albumin (66,200), ovalbumin (45,000), and B-lactoglobulin (18,400). (Lane B) M-2 (b). (Lane C) GMB-1 (a?) + 5940 (a_{0a_2}). (Lane D) GMB-1 (a?). (Lane E) 5940 (a_{0a_2}). (Lane F) 5940 (a_{0a_2}) + WR-5 (a?). (Lane G) WR-5 (a?). (Lane H) WR-5 (a?) + GMB-1 (a?). (Lane I) M-2 (b). B (Lane A) Standard proteins and their molecular weights. From top, phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). (Lane B) 5940 (a_{0a_2}). (Lane C) GMB-1 (a?) + 5940 (a_{0a_2}). (Lane D) GMB-1 (a?). (Lane E) 5940 (a_{0a_2}). (Lane F) GMB-1 (a?) + 5940 (a_{0a_2}). (Lane G) M-2 (b). 1 ug FAg loaded per well (co-electrophoresed samples: 1 ug FAg per sample per well).

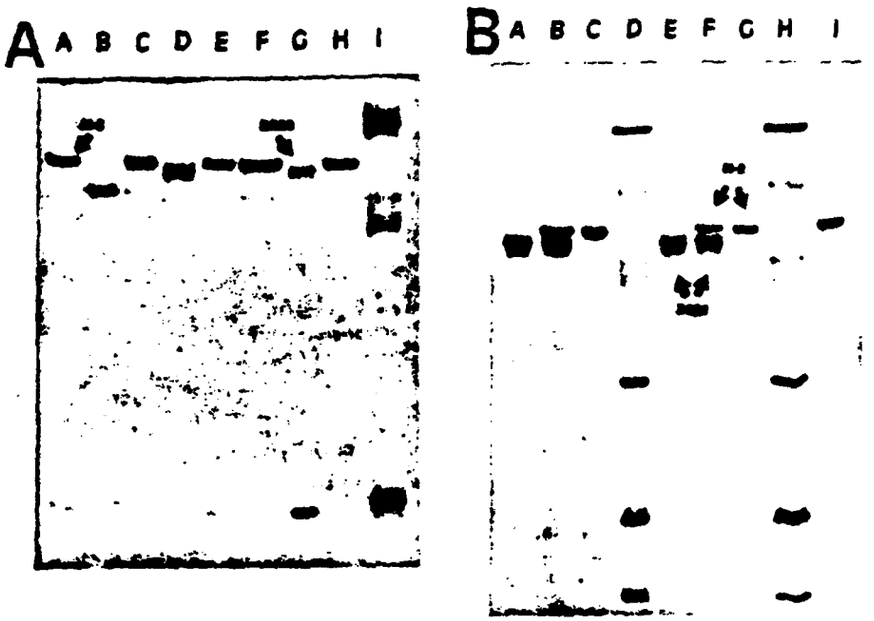


Figure 3. SDS-PAGE of P. aeruginosa PAg preparations. A (Lane A) M-2 (b). (Lane B) 5940 (a_0a_2). (Lane C) 13030 (a_0). (Lane D) 7191 (a?). (Lane E) M-2 (b). (Lane F) 170001 (b). (Lane G) 5933 ($a_0a_1a_2$). (Lane H) M-2 (b). (Lane I) Standard proteins and their molecular weights. From top, bovine serum albumin (66,200), ovalbumin (45,000), and B-lactoglobulin (18,400). B (Lane A) 7191 (a?). (Lane B) 15084 (b) + 7191 (a?). (Lane C) 15084 (b). (Lane D) Standard proteins and their molecular weights. From top, phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). (Lane E) 7191 (a?). (Lane F) M-2 (b) + 7191 (a?). (Lane G) M-2 (b). (Lane H) Standard proteins (repeat). (Lane I) SB1-1 (b). 1 ug PAg loaded per well (co-electrophoresed samples: 1 ug PAg per sample per well).

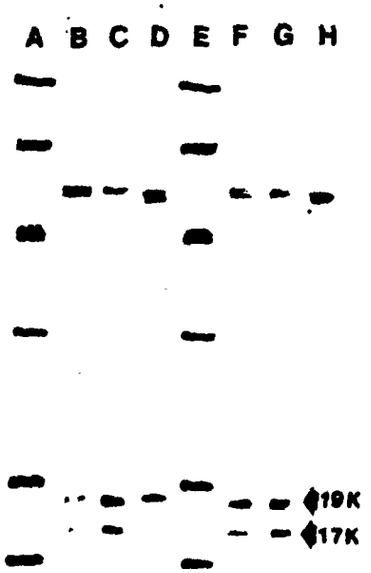


Figure 4. SDS-PAGE of P. aeruginosa PAg preparations. (Lane A) Standard proteins and their molecular weights. From top, phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), and lysozyme (14,400). (Lane B) 1210 ($a_0a_1a_2$) O_2 limited. (Lane C) 1210 ($a_0a_1a_2$) O_2 limited. (Lane D) 1210 ($a_0a_1a_2$). (Lane E) Standard proteins (repeat). (Lane F) 1210 ($a_0a_1a_2$) O_2 limited. (Lane G) 1210 ($a_0a_1a_2$) O_2 limited. (Lane H) 1210 ($a_0a_1a_2$). 1 ug PAg loaded per well.

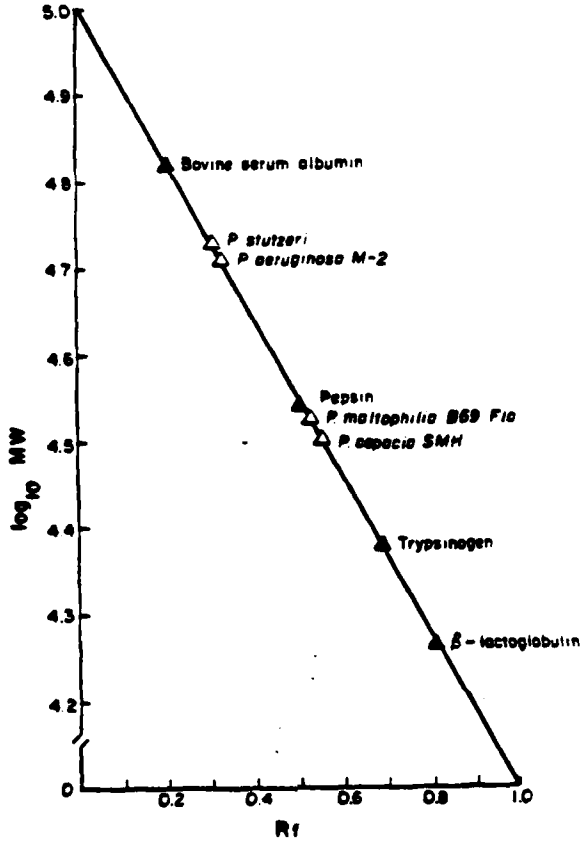


Figure 5. Molecular weight (MW) determinations of Pseudomonas species FAg with SDS-PAGE. Standard proteins: bovine serum albumin (66,200), pepsin (34,700), trypsinogen (24,000) and B-lactoglobulin (18,400).

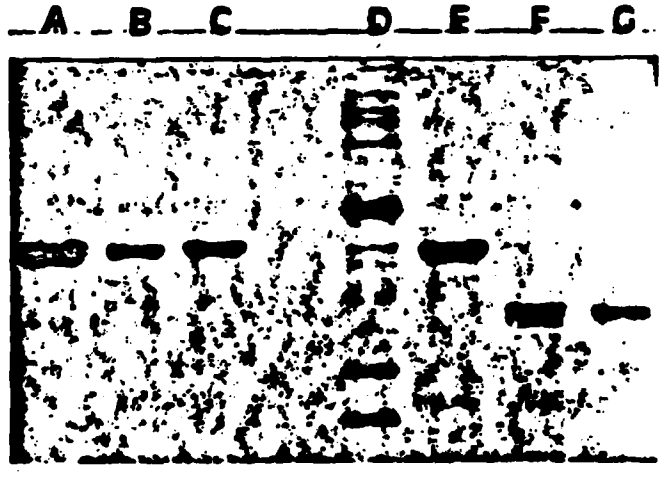


Figure 6. SDS-PAGE of P. cepacia FAg preparations. (Lane A) P. cepacia D7072 Ind. (Lane B) A1560 Fla. (Lane C) 3765 Pa. (Lane D) Standard proteins and their molecular weights. From top, bovine serum albumin (66,000), pepsin (34,700), trypsinogen (24,000), and B-lactoglobulin (18,400). (Lane E) E4119 Co. (Lane F) F3761 PR. (Lane G) SMH.

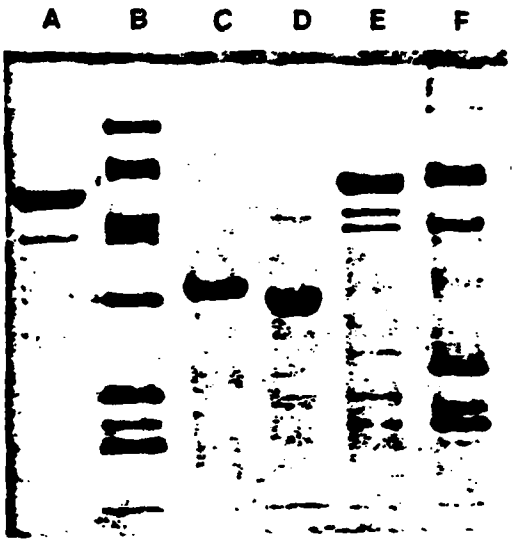


Figure 7. SDS-PAGE of Pseudomonas species FAg preparations. (Lane A) P. aeruginosa M-2. (Lane B) Bio-Rad SDS-PAGE premixed standards, 10,000-100,000 MW. (Lane C) P. maltophilia B69 Fla. (Lane D) P. cepacia BMH. (Lane E) P. stutzeri MEW. (Lane F) Standard proteins and their molecular weights. From top, bovine serum albumin (66,000), pepsin (34,700), trypsinogen (24,000), and B-lactoglobulin (18,400).

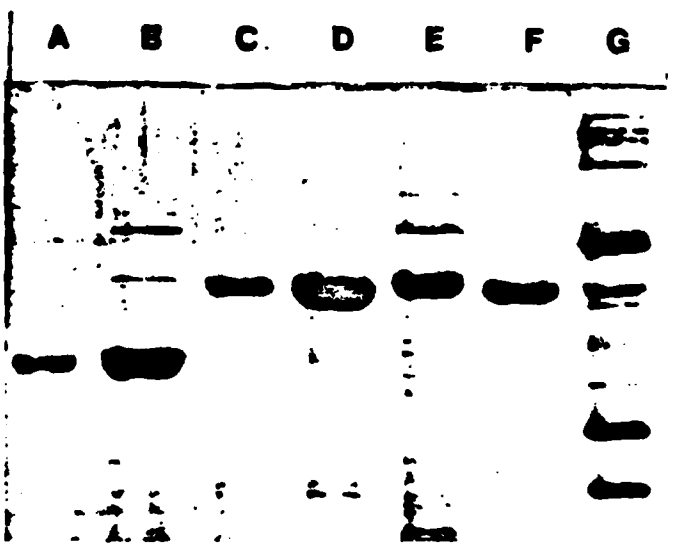


Figure 8. SDS-PAGE of P. cepacia FAg preparations. (Lane A) P. cepacia BMH. (Lane B) E7427 PR. (Lane C) E2973 Ma. (Lane D) E7893 Ill. (Lane E) E8980(1) Col. (Lane F) E4119 Co. (Lane G) Standard proteins and their molecular weights. From top, bovine serum albumin (66,000), pepsin (34,700), trypsinogen (24,000), and B-lactoglobulin (18,400).

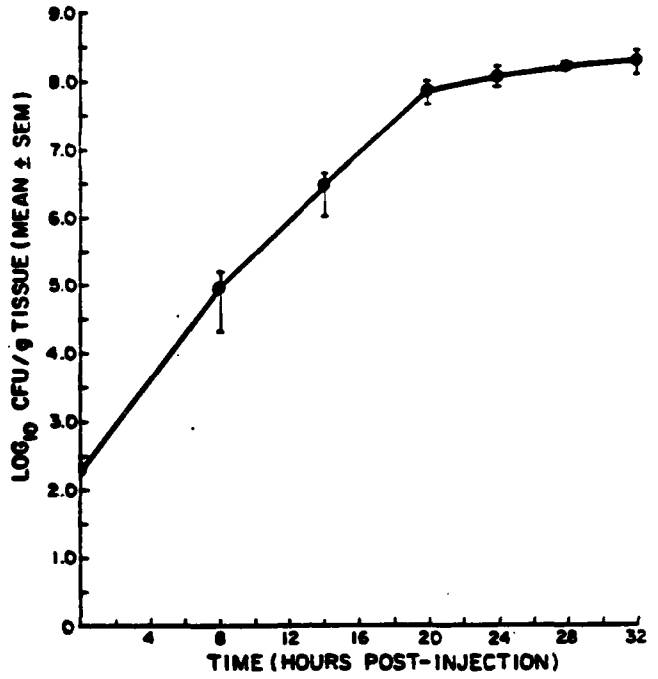


Figure 9. Quantitative bacteriology of burned skin after subcutaneous injection of 1.9 (±0.2) x 10^2 CFU/0.1 ml of P. aeruginosa strain M-2 into the burned skin immediately post-burn.

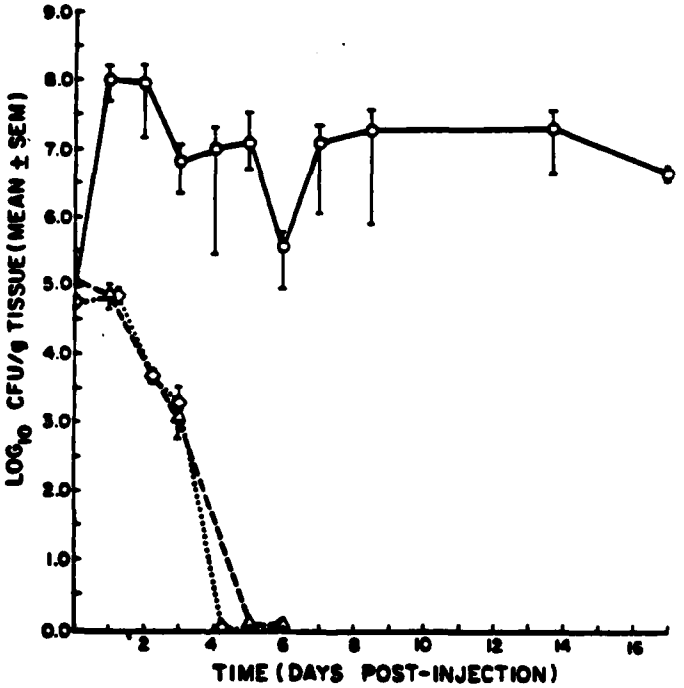


Figure 10. Quantitative bacteriology of skin after subcutaneous injection of P. aeruginosa EMR. Injection of 5.7 (±1.1) x 10^4 CFU/0.1 ml into unburned mice (dotted line). Injection of 1.1 (±0.1) x 10^5 CFU/0.1 ml into unburned skin of burned mice (dashed line). Injection of 1.0 (±0.1) x 10^5 CFU/0.1 ml into burned skin (solid line).

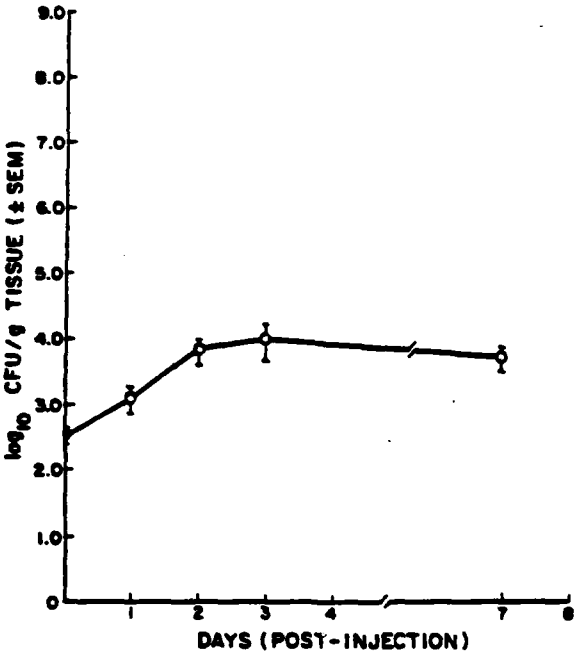


Figure 11. Quantitative bacteriology of burned skin after subcutaneous injection of 3.2 (±0.8) x 10^2 CFU/0.1 ml of P. cepacia SMH into the burned skin.

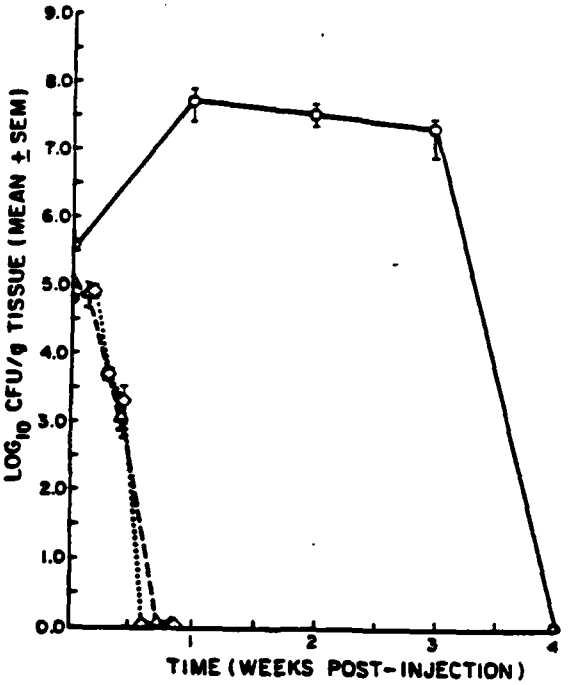


Figure 12. Long term quantitative bacteriology of skin after subcutaneou injection of P. cepacia SMH. Injection of 5.7 (±1.1) x 10^4 CFU/0.1 ml into unburned mice (dotted line). Injection of 1.1 (±0.1) x 10^5 CFU/0.1 ml into unburned skin of burned mice (dashed line). Injection of 3.4 (±0.3) x 10^5 CFU/0.1 ml into burned skin (solid line).

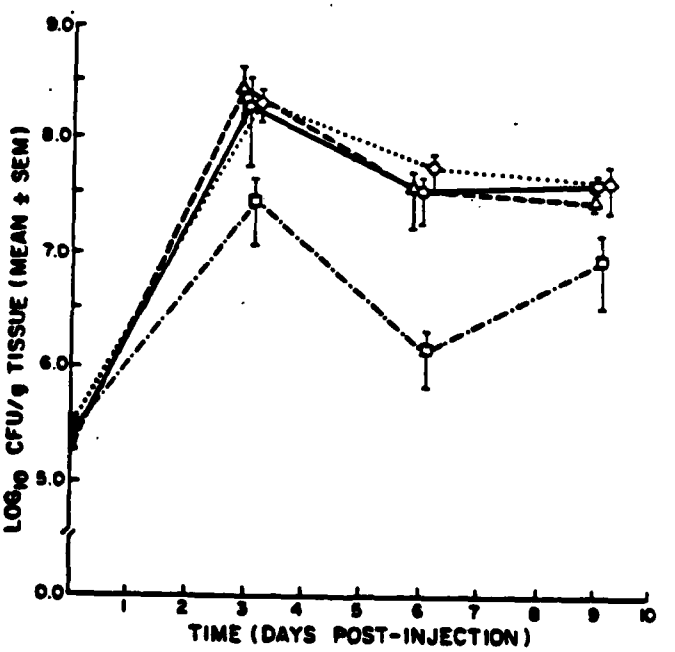
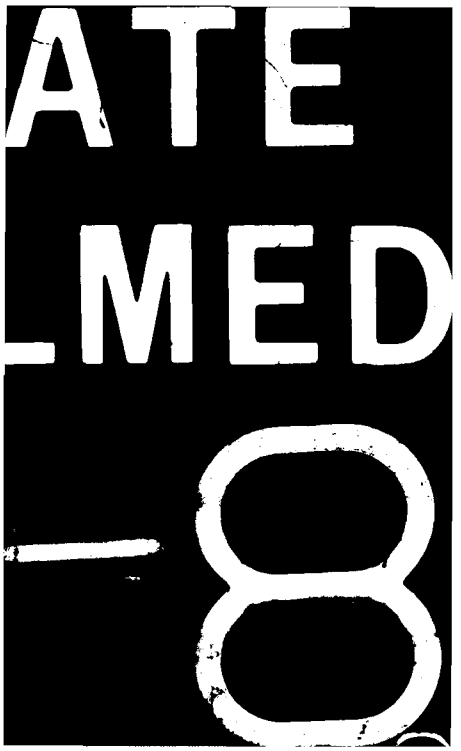


Figure 13. Quantitative bacteriology of skin from immunized mice after subcutaneous injection of P. cepacia SMH (average injection of 2.7 x 10^5 CFU/0.1 ml). Mice were immunized as follows: P. cepacia SB960(1) Col FAg (dashed line); P. aeruginosa M-2 FAg (solid line); P. cepacia SMH FAg (dotted and dashed line); saline control (dotted line).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